The Determination of S-Carboxyethylcysteine and the Cyanoethylation of Milk Proteins

E. B. KALAN, A. NEISTADT, L. WEIL,[1] AND W. G. GORDON

From the Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, U. S. Department of Agriculture, Philadelphia, Pennsylvania

Weil and Seibles (1) demonstrated that complete reduction of the disulfide bonds of the two most abundant whey proteins, α-lactalbumin and β-lactoglobulin, could be accomplished with β-mercaptoethanol. The subsequent alkylation reaction with acrylonitrile was thought to be specifically confined to the thiol groups and to take place quantitatively. The *S*-cyanoethylcysteinyl residues formed in this manner were converted completely to *S*-carboxyethylcysteine upon acid hydrolysis. The method described by Weil and Seibles (1) has the advantages of not requiring any denaturing agent for the two proteins involved and yielding a compound upon acid hydrolysis which can now be easily determined by the automatic method described by Piez and Morris (2).

S-Carboxyethylcysteine was determined by Weil and Seibles (1) in the manual system described by Moore, Spackman, and Stein (3). However, a more recent attempt by Zacharius and Talley (4) to resolve the compound using the automatic system of Spackman, Stein, and Moore (5) resulted in incomplete resolution of the compound from glutamic acid. Plummer and Hirs (6) were also unable to resolve *S*-carboxyethylcysteine, and the present report confirms these findings.

Studies in progress in these laboratories involve the reduction of the disulfide bonds of κ-casein and subsequent stabilization of the thiol groups (7). This method facilitates the preparation of the reduced proteins in a stable form. As a result, it has become of some importance to verify the complete reduction of the disulfide bond and conversion to *S*-cyanoethyl-κ-casein. Therefore a method was developed for the complete resolution and direct estimation of *S*-carboxyethylcysteine based on the findings of Weil and Seibles (1) and utilizing the automatic method of Piez and Morris (2).

[1] Deceased December 7, 1964.

MATERIALS AND METHODS

S-Cyanoethyl-β-lactoglobulin AB and *S*-carboxyethylcysteine were the compounds previously described (1).

S-Cyanoethyl-κ-casein was generously supplied by Dr. J. Woychik of these laboratories. The κ-casein was prepared from pooled milk (7).

L-Serine and L-glutamic acid were commercial products which were ascertained to be chromatographically pure.

The determination of the amino acids, those in synthetic mixtures and those arising from protein hydrolysis, was exactly as described by Piez and Morris (2) except that the column height was 126 cm because of contraction under use and the Varigrad composition was modified as shown in Table 1 to effect resolution with the particular resin employed.

TABLE 1

COMPOSITION OF THE VARIGRAD FOR CHROMATOGRAPHY

Chamber	pH 2.91 buffer, ml	0.8 *M* citrate, ml	H_2O, ml
1	75	0	0
2	75	0	0
3	75	0	0
4	70	5	0
5	50	24	0
6	40	19	15
7	15	59	0
8	15	41	15
9	0	71	0

Single 24-hr hydrolyzates of *S*-cyanoethyl-κ-casein and *S*-cyanoethyl-β-lactoglobulin AB were analyzed using the system of Spackman, Stein, and Moore (5). These experiments were carried out to verify certain findings when the protein hydrolyzates were analyzed in the system of Piez and Morris (2). This will be discussed further.

For the determination of the color constant, peak elution time, peak elution volume, and ratio of the absorbancies at 570 and 440 mμ, a solution of pure *S*-carboxyethylcysteine was made using the pH 2.91 buffer. A solution containing the compound plus L-serine and L-glutamic acid was prepared in a similar fashion to demonstrate the complete resolution of the 3 amino acids.

The stability of *S*-carboxyethylcysteine was determined by hydrolyzing a known quantity in 1.0 ml 6 *N* HCl at 110°C in a sealed, evacuated tube for 24, 72, and 96 hr. Each *S*-cyanoethyl-protein was hydrolyzed for only 24 hr in triplicate in a similar fashion, using about 2.0 mg of

protein. A known quantity of *S*-carboxyethylcysteine was added to one of the samples of each protein before hydrolysis to verify that the compound derived from hydrolysis was identical to the synthetic material. In addition this procedure gave a measure of the recovery of the compound in the presence of both a globular protein and an unusual glycoprotein.

The amino acid composition reported for *S*-cyanoethyl-κ-casein was calculated by finding molar ratios based on 8 different amino acids (Asp, Pro, Ala, Met, Leu, Phe, His, and Arg). The molar ratios from each of the 8 calculations were multiplied by integers selected to yield products of the correct order, that is, numbers of amino acid residues consistent with the molecular weight of the protein. The residue value reported (Table 3) for each amino acid is the average of 24 values derived from three analytical determinations, except for the above 8 amino acids for which 21 values are averaged since these amino acids were arbitrarily taken as one in the calculation of molar ratios. When *S*-carboxyethylcysteine was added before hydrolysis, the quantity added was first subtracted before molar ratios were determined for the *S*-carboxyethylcysteine derived from the protein. Calculations for *S*-cyanoethyl-β-lactoglobulin AB were similar except that 6 amino acids were used to calculate molar ratios: Pro, Leu, Tyr, Phe, His, and Arg.

RESULTS AND DISCUSSION

Figure 1 demonstrates the complete resolution of *S*-carboxyethylcysteine (*S*-Cec) from serine and glutamic acid. The constant or color value for the *S*-carboxyethylcysteine was calculated by the H $\times$ W method of Spackman, Stein, and Moore (5) and found to be 17.40 (average of 6 determinations with a spread of 16.41 to 18.50). In the system employed, leucine has a constant of approximately 27 and the next lower homolog, *S*-carboxymethylcysteine, about 20. The ratio of absorbancies at 570 to 440 mμ calculated from peak heights was found to be 7.13 (average of 18 determinations with a spread of 6.38 to 8.25). For leucine this value is 8.07 and for *S*-carboxymethylcysteine, 6.03. The peak elution time was 311 min (average of 18 determinations with a spread of 298 to 315), the rate of elution being 30 ml/hr; the peak elution volume was 155.5 ml. In no case was any overlapping of peaks observed and quantitation was easily and directly accomplished by the H $\times$ W method.

Table 2 indicates that up to 72 hr of heating in 6 *N* HCl results in the loss of about 8% of the *S*-carboxyethylcysteine. After 96 hr about 11–15% is lost. No new ninhydrin-positive peaks appear on the chromatographic records. However, it is to be noted that, in the experiments in

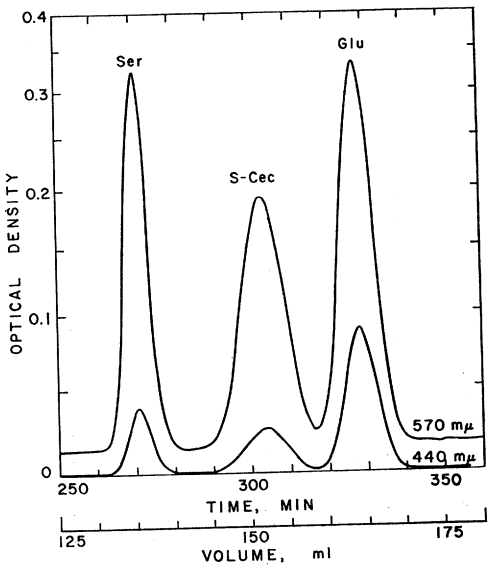

FIG. 1. Chromatogram demonstrating complete separation of a synthetic mixture of *S*-carboxyethylcysteine, serine, and glutamic acid in the system of Piez and Morris (2).

TABLE 2

STABILITY OF *S*-CARBOXYETHYLCYSTEINE TO ACID[a]

Heating time, hr	μmole		% recovery
	Added	Recovered	
24	0.515	0.473	91.8
24	0.515	0.472	91.6
72	0.515	0.472	91.6
96	0.515	0.460	89.3
96	0.515	0.440	85.4

[a] Each sample heated in a sealed evacuated tube with 1.0 ml 6 *N* HCl at 110°C in a circulating-air laboratory oven.

which the compound was added before hydrolysis of the *S*-cyanoethylproteins, there was no apparent loss of *S*-carboxyethylcysteine. For the β-lactoglobulin derivative, 10.4 moles of *S*-Cec and, for the κ-casein derivative, 2.1 moles of *S*-Cec were recovered after deducting the added *S*-Cec. These values are in good agreement with the values of 10 moles of disulfide and thiol sulfur for β-lactoglobulin (8, 9) and 2 moles for κ-casein (7, 10) per mole of respective protein. This would indicate that, up to 24 hr of heating with 6 *N* HCl at 110°C in sealed, evacuated tubes,

S-Cec is protected from destruction by acid in the presence of a protein hydrolyzate.

Table 3 is a comparison of the composition of the *S*-cyanoethyl-proteins with the native proteins. It is seen that the method of reduction fol-

TABLE 3

AMINO ACID COMPOSITION OF *S*-CYANOETHYL-β-LACTOGLOBULIN AB AND *S*-CYANOETHYL-κ-CASEIN: COMPARISON WITH NONALKYLATED PROTEINS

(values based on molar ratios—see text)

Amino acid	*S*-Cyanoethyl-β-lactoglobulin AB[a]	$\pm t_{.05}\bar{S}_{\bar{x}}^{\Delta}$	β-Lactoglobulin AB[b]	*S*-Cyanoethyl-κ-casein[a]	$\pm t_{.05}\bar{S}_{\bar{x}}^{\Delta}$	κ-Casein[a]	$\pm t_{.05}\bar{S}_{\bar{x}}^{\Delta}$
Asp	31.7	0.67	30.3	12.0	0.23	11.7	0.22
Thr[c]	15.5	.34	14.9	11.6	.24	11.2	.22
Ser[c]	12.8	.29	12.5	11.0	.23	10.6	.20
Glu	51.7	.97	49.5	26.2	.55	25.5	.47
Pro	16.8	.38	15.9	17.5	.42	17.2	.38
Gly	7.1	.13	6.8	2.7	.18	2.7	.053
Ala	29.4	.52	28.2	12.8	.32	12.5	.26
1/2 Cys[c]	0	—	10.2	0	—	1.6	.037
Val	18.8	.41	18.5	9.8	.22	9.7	.18
Met	7.5	.15	7.6	1.9	.039	1.9	.048
Ileu	18.2	.39	18.9	11.4	.32	10.5	.20
Leu	43.3	.96	41.8	8.7	.20	8.8	.19
Tyr[c]	7.9	.12	7.7	8.6	.20	8.5	.16
Phe	8.2	.18	7.7	4.2	.095	4.2	.084
Lys	21.9	.48	28.6	5.6	.14	10.0	.22
His	3.8	.13	3.6	2.9	.090	2.9	.071
Arg	6.1	.14	5.8	5.0	.15	5.2	.11
S-Cec	10.4	.21	—	2.1	.12	—	—

[a] See text for conditions of hydrolysis.

[b] Calculated from data in Ref. 8 except for Glu, which is based on data in Ref. 9.

[c] Uncorrected for destruction.

$^{\Delta}$ = Measure of repeatability at 95% confidence limit.

lowed by alkylation with acrylonitrile gives 100% of the disulfide and/or thiol sulfur for both proteins. This is also true for α-lactalbumin (1) as well as for ribonucleases A and B (6). The residue values for *S*-cyanoethyl-β-lactoglobulin AB are somewhat higher than for the native protein. This may be due to the difference in calculations for the two proteins—the former by the method of molar ratios, the latter by percentage composition based on a molecular weight of 36,000. Nevertheless the compositions are similar except for the case of lysine, in which a reduction of about 25% is observed for the *S*-cyanoethyl-protein. Similarly, the κ-caseins are also alike in composition except for lysine, which is reduced by about 45% for the *S*-cyanoethyl-protein. A recalculation

of the data previously reported for *S*-cyanoethyl-α-lactalbumin (1) has revealed a reduction of lysine content of 11%. Therefore, it seems apparent that, for the milk proteins investigated, alkylation with acrylonitrile is not entirely specific under the conditions employed in that some lysine residues react. In addition, Levin and Stepanov (11) have found that cyanoethylation of reduced hog pepsin results in the modification of about 64% of the amino groups of the *N*-terminal leucine. However, Plummer and Hirs (6) have reported that acrylonitrile is specific for thiol groups under specified conditions after reduction of ribonucleases A and B. These investigators have demonstrated that, after reduction of ribonucleases A and B, an exposure time of the reduced protein to acrylonitrile from 2 to 4 hr at pH 8 has no influence on the composition of the product, but that modification of lysine residues occurs when cyanoethylation is performed at pH 9.5 (6).

Figure 2 is a chromatogram of a hydrolyzate of *S*-cyanoethyl-κ-casein, exclusive of the basic amino acids. This provides additional evidence

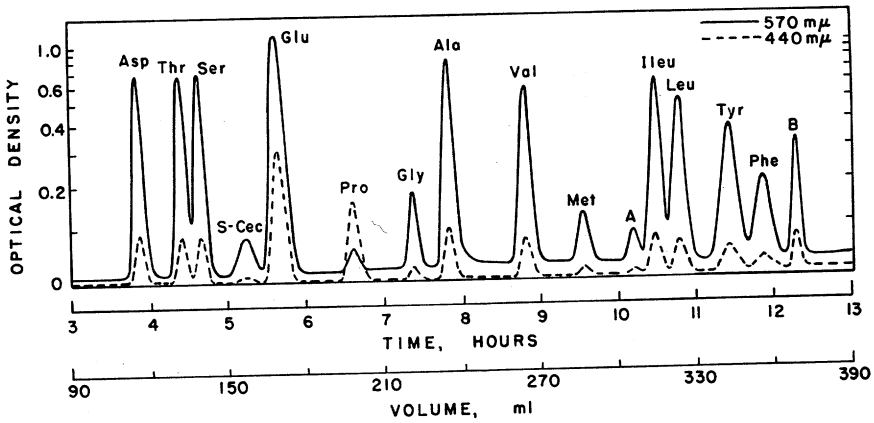

FIG. 2. Chromatogram of 24-hr acid hydrolyzate of *S*-cyanoethyl-κ-casein demonstrating resolution of *S*-carboxyethylcysteine, absence of cystine, and appearance of two new peaks, A and B. System of Piez and Morris (2).

of the nonspecificity of alkylation with acrylonitrile and the completeness of the reaction with respect to thiol groups. It can be seen that there is a total absence of cystine which normally occurs between alanine and valine. Instead, a new peak, *S*-Cec, occurs between serine and glutamic acid, completely resolved and accounting for the total disulfide content of κ-casein. In addition, a prominent new peak, B, which occurs immediately following phenylalanine, is presumed to be related to the decrease in lysine. This peak is of sufficient area to account for the

loss of lysine, and has the characteristics shown in Table 4, with respect to its elution behavior in the system of Piez and Morris (2). A second peak, A, is eluted at earlier times and is also probably related to the

TABLE 4

ELUTION CHARACTERISTICS OF UNKNOWN COMPOUNDS RESULTING FROM HYDROLYSIS OF *S*-CYANOETHYL-PROTEINS[a]

Peak[b]	$\frac{570\ m\mu}{440\ m\mu}$	Peak elution time, min	Peak elution volume, ml
A	7.42 (6.90–8.00)	612 (609–615)	306 (304.5–307.5)
B	4.44 (4.37–4.58)	736 (733–738)	368 (366.5–369)

[a] Each value is the average of six determinations with the spread shown in parentheses. System of Piez and Morris (2).

[b] The ratio of absorbancies at 570 and 440 mμ for peaks A and B are 6.0 and 4.2, respectively (average of 2 determinations), in the system of Spackman, Stein, and Moore (5).

decrease in lysine. It occurs immediately before isoleucine, and its elution properties are included in Table 4. Peak A cannot be *allo*-isoleucine based on the discussion below. It is likely that peaks A and B, presumably derived from lysine, represent mono- or disubstituted products, alkylated at the ε-amino group of the amino acid.

In order to ascertain that the new peaks, A and B, were not artifacts of the chromatographic procedure, a 24-hr hydrolyzate each of *S*-cyanoethyl-β-lactoglobulin AB and of *S*-cyanoethyl-κ-casein was analyzed in the system of Spackman, Stein, and Moore (5). Figure 3 is a chromatogram of the acidic, neutral, and aromatic amino acids as eluted from

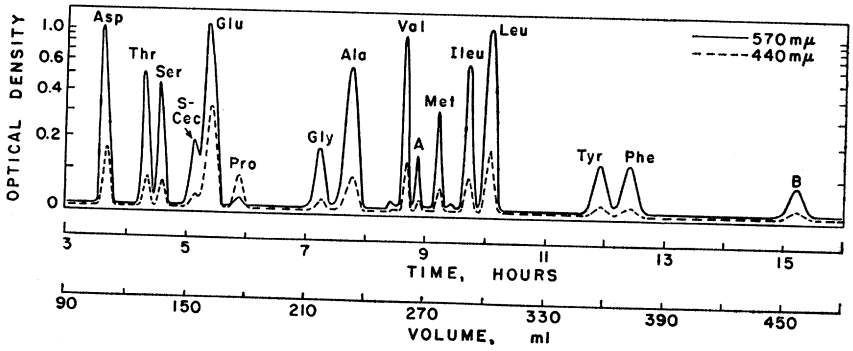

FIG. 3. Chromatogram of 24-hr acid hydrolyzate of *S*-cyanoethyl-β-lactoglobulin AB demonstrating lack of resolution of *S*-carboxyethylcysteine, absence of cystine, and appearance of two new peaks, A and B. System of Spackman, Stein, and Moore (5).

the 150-cm column. It is abundantly clear that *S*-carboxyethylcysteine is incompletely separated from glutamic acid in this system, confirming the findings of Zacharius and Talley (4) and Plummer and Hirs (6). Two new peaks appear, again designated A and B, and believed to correspond to the same lettered peaks in Fig. 2. If this is true, then peak A cannot be *allo*-isoleucine since, in the system of Spackman, Stein, and Moore (5), *allo*-isoleucine occurs between methionine and isoleucine. It is interesting to note that peak A seems to be better resolved in this system than in the system of Piez and Morris (2). Peak B occurs some 150 min after phenylalanine in Fig. 3, and the considerable length of time for its elution may account for the fact that previous investigators (1) have not reported its occurrence. However, Fig. 3 clearly confirms the findings demonstrated in Fig. 2 and makes it highly unlikely that peaks A and B are artifacts of the chromatographic procedure.

Finally, it would appear that the varying extent of the alkylation of reduced proteins at sites other than thiol groups, in particular lysine, depends on both the nature of the protein and the conditions of alkylation with acrylonitrile. This is seen in the fact that nonspecific alkylation of lysine residues occurs to the extent of about 45% in κ-casein, 25% in β-lactoglobulin, 11% in α-lactalbumin, and not at all in ribonucleases A and B. About 64% of the amino groups of *N*-terminal leucine are modified in hog pepsin.

SUMMARY

The automatic method described by Piez and Morris (2) has been used with slight modifications to effect a complete resolution of *S*-carboxyethylcysteine. This allows for the direct quantitation of the compound and facilitates the use of its protein precursor, *S*-cyanoethylcysteine, in compositional and structural studies. It has been demonstrated that 100% of the disulfide and/or thiol groups of proteins can be determined by alkylation of the reduced proteins with acrylonitrile, followed by acid hydrolysis. It has also been shown that acrylonitrile is a nonspecific alkylating agent for at least three milk proteins under specified conditions and that up to 45% of the lysine residues may be alkylated. Further, for *S*-cyanoethyl-β-lactoglobulin AB and for *S*-cyanoethyl-κ-casein, at least two new ninhydrin-positive peaks occur in two different chromatographic systems after acid hydrolysis of the proteins. These peaks are presumed to be related to the decrease in lysine.

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